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Title: Method for Improving Genetic Stability)  
of Foreign Insert Nucleotide Sequence in)  
Recombinant single-Stranded RNA Virus)

**STATEMENT OF  
ACCURACY**

Hon. Commissioner of Patents  
and Trademarks  
Washington, D.C. 20231

Sir:

The undersigned hereby states that the attached English language patent application is an accurate translation of *Korean* patent application filed on February 8, 2001 and assigned Serial No. 2001-6229.

Date: August 27, 2003

Signed: 

Printed Name: Boo-Hyun YANG

1

**[ABSTRACT]****[ABSTRACT]**

The present invention relates to methods for improving a genetic stability of an insert in a single-stranded RNA virus, more particularly, to methods for improving a genetic stability of recombinant live vaccine derived from poliovirus vector, which comprises performing a mutagenesis of the foreign insert nucleotide sequence to provide even distribution of G/C content throughout the overall foreign DNA sequence and synthesizing the insert DNA sequence around 500 bp by ligation-free PCR method without template. The present method provides a vector comprising more various antigenic determinant sites compared to the conventional poliovirus vector technology and a method for improving significantly a genetic stability of a foreign sequence, thereby permitting to enforce the applicability of poliovirus vector and single-stranded RNA virus as recombinant live vaccine.

**20 [REPRESENTATIVE FIGURE]**

Fig. 11a

**[KEY WORDS]**

single-stranded RNA virus, poliovirus, Sabin type 1,  
25 recombinant vector, genetic stability, G/C content

## [SPECIFICATION]

## [TITLE OF THE INVENTION]

Method for Improving a Genetic Stability for the  
5 Insert in Single-Stranded RNA Virus Recombinant Vectors

## [BRIEF DESCRIPTION OF THE DRAWINGS]

Fig. 1 represents a genetic map of RPS-Vax vector used  
in this invention;

10 Fig. 2 shows the procedures for preparing chimeric  
poliovirus according to this invention;

Fig. 3a is a photograph showing RT-PCR analysis  
demonstrating a genetic stability of PV 2-118 integrated  
into RPS-Vax;

15 Fig. 3b is a photograph showing RT-PCR analysis  
demonstrating a genetic stability of PV 2.3-131 integrated  
into RPS-Vax;

Fig. 3c is a photograph showing RT-PCR analysis  
demonstrating a genetic stability of HIV-1 mV3 integrated  
20 into RPS-Vax;

Fig. 4a represents the characteristics of HIV-1 env-98,  
HIV env-83, HIV-1 env-71 and HIV-1 env-98/M sequences.

Fig. 4b shows the characteristics of a portion in HIV-  
1 env-98/M subject to mutagenesis according to the rule of  
25 this invention;

Fig. 4c is a graph representing the distribution of  
G/C content of HIV-1 env-98 and HIV-1 env-98/M;

3

Fig. 5 is a photograph showing RT-PCR analysis demonstrating a genetic stability of HIV-1 env-98, HIV-1 env-83, HIV-1 env-71 and HIV-1 env-98/M integrated into RPS-Vax;

5 Fig. 6a is a photograph showing RT-PCR analysis demonstrating a genetic stability of SIV p27-150 and SIV p27-167 integrated into RPS-Vax;

Fig. 6b is a photograph showing Western blot analysis demonstrating a protein stability of SIV p27-150 or SIV  
10 p27-167 integrated into RPS-Vax;

Fig. 7 is a photograph showing RT-PCR results demonstrating in vivo genetic stability of SIV p27-150 or SIV p27-167 in RPS-Vax administered into Tg-PVR mice after 1-4 days;

15 Fig. 8 represents the characteristics of OPV-150 sequence prepared in accordance with this invention;

Fig. 9 shows 8 primers and their characteristics used for ligation-free PCR of the DNA sequence encoding OPV-150;

20 Fig. 10 is a photograph showing product from ligation-free PCR of the DNA sequence encoding OPV-150;

Fig. 11a is a photograph showing RT-PCR analysis demonstrating a genetic stability of OPV-150 integrated into RPS-Vax; and

25 Fig. 11b is a photograph showing Western blot analysis demonstrating a protein stability of OPV-150 integrated into RPS-Vax.

## [DETAILED DESCRIPTION OF THE INVENTION]

## [OBJECTS OF THE INVENTION]

## [FIELD OF THE INVENTION AND PRIOR ARTS]

5       The present invention relates to methods for improving a genetic stability of a foreign insert DNA in a single-stranded RNA virus, more particularly, to methods for preparing a single-stranded RNA virus exhibiting improved genetic stability, particularly, to methods for  
10 preparing a recombinant live vaccine by use of poliovirus vector with improved genetic stability.

Live attenuated viral vaccines have been reported to have several advantages over other types of vaccines: low  
15 cost for production, higher immunogenicity, and easy for administration. However, the greatest advantage has been offered by the well-characterized molecular structures of target viruses which enable investigators to manipulate the viral cDNA genome with a recombinant DNA technique  
20 even with RNA viruses, as to produce recombinant progeny viruses (Rolph, M. S. and I. A. Ramshaw., *Curr. Opin. In Immunology* 9:517-524(1997)). The principal idea is to insert the exogenous insert nucleotide sequence encoding the desired foreign antigen into the attenuated viral  
25 genome without altering the viability of the virus. The recombinant thus prepared can be served as a complex vaccine to induce the immune responses to the introduced

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vaccine gene as well as the vector. Theoretically, recombinant viruses can be used as an efficient recombinant vaccine, since the inserted genes can be replicated, expressed and processed along with the viral genome, subsequently leading to induce immune responses not only to the parental viruses but also to the introduced foreign antigens. The utility of this vaccine approach, however, has been largely constrained by several factors such as a limitation of an insert size, far reduced replication capacity, genetic instability at passages, or a recurrence of the pathogenicity of the parental or recombinant viruses.

Many attempts have been made to manipulate the poliovirus as a favorable vaccine vector because of its attractive characteristics of safe usage, low cost, convenient administration, and long-lasting protective immunity in both mucosal and systemic immune responses, which have been established for decades. However, the poliovirus has a smaller genome than those of other viruses, so that the acceptable size of the foreign gene is very likely to be restricted as used for vector. Such fact is considered one of the most serious obstacles for a wide application of recombination poliovirus as an effective live viral vaccine vector.

Poliovirus, as a member of *Picornaviridae*, is a nonenveloped, (+)-single-stranded RNA virus containing 7.44 kb of RNA genome. The poliovirus shows a high

incidence of genetic mutation due to error-prone replication mechanism and multiple genetic recombination between progeny RNAs. Its genome contains an internal ribosomal entry site (IRES) followed by a single open  
5 reading frame (ORF) encoding a long polyprotein. The IRES element controls the expression of the polyprotein that is subsequently cleaved into several structural and nonstructural proteins by three kinds of virus-encoded proteases (2A<sup>pro</sup>, 3C<sup>pro</sup>, and 3CD<sup>pro</sup>). A major viral protease,  
10 3C<sup>pro</sup>, and its precursor, 3CD<sup>pro</sup>, cleave the polyprotein at a specific site (AXXQ/G) within the expressed polyprotein. A minor protease, 2A<sup>pro</sup>, cleaves the polyprotein at the junction between the P1 and P2 regions. Exactly 60 copies of each of four different capsid proteins (VP1, VP2, VP3,  
15 and VP4) are assembled into a rigid icosahedral viral capsid that concomitantly encapsidates the viral genome. Since the expression, cleavage, replication and assembly of the poliovirus described above are spontaneous, so that a complete virus can be proliferated rapidly in acceptable  
20 host cells.

The polyprotein fusion strategy, one of the strategies for poliovirus-based vaccine developments, was directed at fusing the foreign insert to either at N-terminus or at the junction between the capsid proteins and nonstructural  
25 proteins (P1/P2) in the long polyprotein with an artificial cleavage site for poliovirus-specific proteases (Andino, R., D. et al., *Science* 265:1448-1451(1994) and

7

U.S. Pat No. 5,965,124). Accordingly, the foreign insert is cleaved-off by one of proteases and remains as a free form in the cytoplasm after being translated together with the viral proteins. A number of the recombinant polioviruses constructed by this strategy were demonstrated for their humoral, cellular, or mucosal immunogenicity against introduced exogenous antigens (Crotty, S., et al, *J. Virol.* 73:9485-9495(1999); and Mandl, S. et al., *Proc. Natl. Acad. Sci, USA* 95:8216-8221(1998)).

However, the plausibility of this strategy was challenged by the proliferation capacity and the genetic instability of the recombinant viruses (Mueller, S., and E. Wimmer,, *J. Virol.* 72:20-31(1998)). Nevertheless, a clear molecular mechanism controlling insert stability has not been well established and the results so far reported have suggested that the introduction of foreign insert larger than 10 kDa into poliovirus capsid is very difficult.

Throughout this application, various patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention pertains.



**[TECHNICAL PROBLEMS TO BE SOLVED BY THE INVENTION]**

To be free from shortcomings of the poliovirus vector systems, the present inventors have researched and as a result found that the foreign DNA sequence subject to  
5 adjustment to have increased G/C content and even G/C distribution shows improved genetic stability in the live vaccine prepared by use of poliovirus vector, and the insert of up to 17 kDa could be stabilized.

Accordingly, it is an object of this invention to  
10 provide a method for improving a genetic stability of a foreign insert DNA sequence in a recombinant single-stranded RNA virus vector.

It is another object of this invention to provide a method for improving a genetic stability of a foreign  
15 insert DNA sequence in a recombinant poliovirus vector.

It is still another object of this invention to provide a method for preparing a DNA molecule using ligation-free PCR method.

It is further object of this invention to provide a  
20 recombinant single-stranded RNA virus and a recombinant poliovirus vector containing a foreign DNA sequence with improved genetic stability.

**[ELEMENTS AND OPERATIONS OF THE INVENTION]**

25 The present invention is directed to a method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant single-stranded RNA

virus vector, which comprises performing a mutagenesis of the foreign insert nucleotide sequence to provide even distribution of G/C content throughout the overall foreign DNA sequence.

5 In another aspect of this invention, there is provided a method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant poliovirus vector, which comprises performing a mutagenesis of the foreign insert nucleotide sequence to provide even  
10 distribution of G/C content throughout the overall foreign DNA sequence.

The present invention will be described in more detail as follows:

15 The present inventors had developed a poliovirus vaccine vector system with various applications (Korean Pat. Appln. No. 1998-32198) and have then accomplished this invention for improving a genetic stability of a live vaccine prepared by inserting foreign DNA into the vector  
20 system developed.

Meanwhile, to accomplish the substantial application of poliovirus vector, some matters should be considered. First of all, there has been a safety concern in the use of poliovirus vector delivery vector for human, since live  
25 agents can possibly undergo genetic reversal from the attenuated to its wild genotype in the progress of passage. The potential hazard might be avoided by using exclusively

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Sabin type 1 poliovirus strain. It has been known that this strain, unlike other attenuated strains of poliovirus, contains compound mutations compared to the wild type sequence, and hence has rare incidence to revert to a pathogenic form. Sabin type 1 poliovirus has been widely used for oral vaccine throughout the world after the year of 1961 and has been reported to show no adverse effects. On contrary to, OPV containing Sabin types 1, 2 and 3 has been reported to cause 5-10 cases of polio per year in USA, which has been revealed to be ascribed to the reversion of Sabin types 2 and 3 to the wild type due to mutation. Therefore, several developed countries including the USA has recently changed the inoculation program for polio in such a manner that oral live vaccine (OPV) is replaced by inactivated vaccine (IPV) or oral live vaccine is administered after IPV administration. The present inventors have constructed vaccine vectors by manipulation Sabin type 1, which have been filed for patent application (Korean Pat. Appln. No. 1998-32198, U.S.Pat. Appln. No. 09/284,349, Canadian Pat. Appln. No. 2.268.737, Japanese Pat. Appln. No. pyeong11-511995, Chinese Pat. Appln. No. 98801323.1, European Pat. Appln. No. 89 938 993.7, Brazil Pat. Appln. No. PI9806084-8). By using it, recombinant viruses containing various antigenic domains have been produced and revealed to express the insert DNA in proliferation. As a result, the vector has been suggested to elicit mucous immune response (Jung, H., and Y.-S. Bae.,

*J. Biochem. Mol. Biol.* 31:432-443(1998)).

The second consideration lies in the genetic stability of insert DNA. The molecular mechanism governing the stable passage of the insert DNA was not well established.

5 The genetic stability of foreign insert DNA has been predicted with difficulty from the sequence and had to be determined empirically. It has been regarded that the application of the single-stranded recombinant RNA virus including vaccine would be much expedited if the stability

10 of the given insert DNA could be assessed prior to the experimental onset. The problems associated with genetic stability of inset DNA are not limited to recombinant poliovirus vector but applied to recombinant single-stranded recombinant RNA virus vectors. RNA viruses are

15 replicated using RNA-dependent RNA polymerase in host cells, and the enzyme is error-prone unlike DNA polymerase to produce various viruses mutated. Where the recombinant vector is prepared using DNA obtained from RNA virus and inset DNA, viruses with various deleted forms in region

20 beyond the size of viral genome are very likely to be produced. While viruses having genome with deletion are also produced, they disappear by natural selection associated with poor proliferation. Therefore, viruses selectively produced show deleted insert DNA unaffected

25 virus growth rate. Although the methods described in this application have particularly been developed to enhance genetic stability of inset DNA in recombinant poliovirus

12

vector, they may be applicable in order to overcome genetic instability associated with the development of recombinant vector using cDNA of single-stranded RNA virus.

For this purpose, the inventors have examined  
5 potential factors governing stability within the insert foreign gene, using recombinant polioviruses constructed with a series of different antigens.

The term used herein "genetic stability of virus  
10 (recombinant) vector" refers to that the insert DNA sequence integrated into a viral vector is stably maintained. The term "genetic stability" refers to that the insert DNA sequence is stably maintained during consecutive passage of single-stranded RNA virus  
15 recombinant vector, in particular, recombinant poliovirus vector so that the immune reaction against a given antigenic determinant site encoded by the insert is elicited. The term used herein "passage stability" is the same meaning as genetic stability. The term "protein  
20 stability" is used to express the genetic stability in protein level. The genetic stability include, in a broader sense, protein stability encoded by the insert sequence.

According to the invention, the genetic stability of  
25 the foreign insert DNA sequence integrated into a poliovirus recombinant vector is accomplished by performing a mutagenesis of the foreign DNA sequence to

provide even distribution of G/C content throughout the overall foreign insert DNA sequence. The mutagenesis should not lead to a substantial change of amino acid sequences encoded by the insert DNA. In particular, if the  
5 foreign insert codes for a polypeptide or a protein covering antigenic determinant sites, the mutagenesis should not be substantially detrimental to its antigenicity.

The enhancement of genetic stability is accomplished  
10 by allowing for even distribution of G/C content and increasing G/C content to above 40% in the insert DNA sequence.

As demonstrated in Examples below, local A/T-rich  
15 region in the insert DNA sequence causes structural instability of RNA transcript derived from the insert DNA sequence, thereby promoting the site-specific deletion of the neighboring region of the insert. Therefore, the mutation for providing even distribution of G/C content in  
20 the foreign DNA sequence is accomplished by increasing G/C content in local A/T-rich region and avoiding A/T-rich region.

It is general that a higher G/C content avoids local A/T-rich region. It could be appreciated that the G/C  
25 content around 40-50% is enough to provide the genetic stability of insert. Therefore, the G/C content more than 50% completely removes the occurrence of local A/T rich

14

sequence. The critical matter lies in the avoidance of local A/T rich sequence by even distribution of G/C content rather than the level of G/C content. In a preferred embodiment, the insert DNA sequence carried in poliovirus recombinant vector has a size of less than 480 bp, more preferably, less than 460 bp and most preferably, less than 450 bp. If the size of insert DNA exceeds 450 bp, the genetic stability of insert is liable to be decreased although it has the G/C content more than 40%. It is assumed that the size limitation is ascribed to a limited packaging capacity of virus. The size of insert, 450 bp corresponds to 6% of the overall genome of poliovirus and may be considered the upper size limit.

According to this invention, it is preferred that the mutagenesis to enhance the genetic stability of the insert DNA sequence cause no substantial amino acid substitutions. In a preferred embodiment, the mutagenesis is performed using codon degeneracy by silent mutation.

The RNA transcript derived from the insert DNA manipulated according to this invention is likely to have compact RNA conformation and be readily encapsidated into rigid virus capsid.

The recombinant poliovirus vector is derived from poliovirus including poliovirus type 1 (Mahoney),

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poliovirus type 2 (Lansing) and poliovirus type 3 (Leon). It is more advantageous that the poliovirus is a live attenuated strain including poliovirus Sabin type 1, poliovirus Sabin type 2 and poliovirus Sabin type 3. Most preferably, the poliovirus is poliovirus Sabin type 1 which has been reported to show the lowest incidence of back mutation to a pathogenic wild type.

The most prominent utility of the poliovirus recombinant vector is an application to providing a vaccine vector. Therefore, the foreign insert nucleotide sequence includes the nucleotide sequence encoding an antigenic determinant site of an infectious virus including HIV-1, HIV-2, HCV, HBV, HPV (human papilloma virus), HSV (herpes simplex virus), rotavirus, influenza virus and epidemic hemorrhagic fever virus, but not limited to.

In the present invention, the foreign DNA sequence integrated into poliovirus recombinant vector is inserted into its 5'-end. This strategy is disclosed in Korean Pat. Appln. No. 1998-32198 filed by the inventors in which the protein encoded by the foreign DNA sequence is fused to the N-terminal of multiple protein. More preferably, the foreign DNA sequence is inserted into a cloning site containing at least one restriction cleavage site artificially formed at the 5'-end of the recombinant



16

poliovirus vector. It is preferred that the cloning site is a multiple cloning site. Preferably, the cloning site further comprises at its 3'-direction a protease cleavage site recognized by endogenous protease, more preferably 3C-protease encoded by a poliovirus genome.

It would be recognized by one skilled in the art that the method for enhancing the genetic stability of live vaccine using poliovirus vector may be applicable to other vectors derived from single-stranded RNA virus to which poliovirus belongs. In other words, the insert DNA sequence integrated to single-stranded RNA virus vector can be manipulated according to the methods described above so as to enhance the genetic stability of the insert DNA sequence. The single-stranded RNA virus vector includes a poliovirus recombinant vector, a yellow fever virus vector, a Venezuelan equine encephalitis virus vector, a rubella virus vector and a Cocksackie virus vector, but not limited to.

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In still another aspect of this invention, there is provided a method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant Sabin type 1 poliovirus vector comprising (a) a genomic nucleotide sequence of poliovirus; (b) a cloning site containing restriction cleavage sites artificially formed at the 5'-end of the genomic nucleotide sequence; and (c)

17

a 3C-protease cleavage site formed between the genomic nucleotide sequence and the cloning site, which comprises performing a mutagenesis of the foreign DNA sequence inserted into the cloning site in order to provide even  
5 distribution of G/C content throughout the overall foreign DNA sequence and to have the G/C content of more than 40%.

The poliovirus genome included in the recombinant poliovirus vector includes the genomes of Mahoney (type 1),  
10 Lansing (type 2), Leon (type 3), Sabin type 1, 2 and 3 and other poliovirus variants with mutation not detrimental to viability of poliovirus.

It is general that a multimeric antigenic determinant  
15 site (epitope) having repetitive amino acid sequences is constructed to enhance antigenicity of vaccine. However, the repeated nucleotide sequence encoding the multimeric epitope is liable to deletion in the course of passage.

To enhance the genetic stability of multimeric epitope  
20 in poliovirus vector, there is provided a method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant Sabin type 1 poliovirus vector comprising (a) a genomic nucleotide sequence of poliovirus; (b) a multiple cloning site  
25 containing restriction cleavage sites artificially formed at the 5'-end of the genomic nucleotide sequence; and (c) a 3C-protease cleavage site formed between the genomic

nucleotide sequence and the cloning site, which comprises performing a mutagenesis of the foreign DNA sequence which is inserted into the multiple cloning site and encodes a multimeric antigen determinant site containing repeated amino acid sequences in order to provide even distribution of G/C content throughout the overall foreign DNA sequence and to avoid repeated nucleotide sequences by use of degeneracy of genetic code.

Where the poliovirus vector prepared by the present method is employed for vaccine applied to human, it is preferred that the DNA sequence encoding multimeric antigenic determinant site is prepared according to the following rules: first, avoid the less used codon in human as a host for poliovirus, second, use the high G/C content among the highly preferred ones, and third, minimize the local repeat such as A/T rich sequence for the whole insert.

The DNA sequence encoding repeated antigenic determinant site may be obtained by the ligation-free PCR method. Accordingly, in further aspect of this invention, there is provided a method for amplifying a DNA sequence using a ligation-free PCR method, which comprises the steps of: (i) preparing a plurality of DNA fragment serving as both template and primer and having the following characteristics: (a) the plural primers are designed by dividing the entire DNA sequence of interest into several fragments into several fragments with

19

suitable size; (b) the 5'- and/or 3'-ends of the plural primers comprise a complementary sequence to the terminal sequence of other primer; (ii) mixing the primers such that the primers corresponding to both ends which is used  
5 in amplification step has a higher concentration than the other primers; (iii) preparing a full length of the foreign DNA sequence with desired size encoding multimeric antigen determinant site by PCR for 20-40 sec at 92-96°C (denaturation), for 20-40 sec at 25-40°C (annealing) and  
10 for 30-55 sec at 68-75°C (extension); and (iv) amplifying the prepared full length of the foreign DNA sequence by PCR for 20-40 sec at 92-96°C (denaturation) and 40 sec - 1 min 10 sec at 68-75°C (annealing and extension).

Intermediates even though generated in maturation step  
15 are rarely amplified in final amplification step, due to a higher annealing temperature and a shortage of internal primers in concentration as compared to those of external primers that are complementary to both ends of the PCR product, respectively. Therefore, according to the present  
20 method, the nucleotides sequence of interest can be yielded as a sole final product without adding template DNA and ligation step.

According to preferred embodiment, the concentration ratio of the primers corresponding to both ends of final  
25 PCR product to the other primers is 1:3-1:8, more preferably, 1:3-1:6 and most preferably, 1:4.

In preferred embodiment, the complementary regions of

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the junctional primers have 8-20 bp in length and G/C content of more than 35% and more preferably, 10-17 mer of length and G/C content of more than 40%. It is preferred that the primers corresponding to both ends of the final  
5 product have a cloning site consisting of restriction enzyme sites.

Where the ligation-free PCR method is applied to the preparation and amplification of the DNA sequence encoding  
10 repetitive antigenic determinant site, a plural of DNA fragments serving as both template and primer have the following characteristics: (a) the plural primers are designed by dividing the entire DNA sequence of interest into several fragments into several fragments with  
15 suitable size; (b) the 5'- and/or 3'-ends of the plural primers comprise a complementary sequence to the terminal sequence of other primer; (c) the complementary sequence at the the 5'- and/or 3'-ends of the primers is 8-20 mer in size and has G/C content of more than 35%; and (d) the  
20 primers corresponding to both ends in amplification step have a cloning site containing restriction cleavage site. Thereafter, the primers are mixed such that the concentration ratio of the primers corresponding to both ends in amplification step to the other primers is 1:3-1:8.  
25 Then, for a maturation step, a full length of the foreign DNA sequence with desired size encoding multimeric antigen determinant site is prepared by PCR for 20-40 sec at 92-

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96°C (denaturation), for 20-40 sec at 25-40°C (annealing) and for 30-55 sec at 68-75°C (extension). Finally, the prepared full length of the foreign DNA sequence is amplified by PCR for 20-40 sec at 92-96°C (denaturation) and 40 sec - 1 min 10 sec at 68-75°C (annealing and extension).

Consequently, where the method for genetic stability and ligation-free PCR method are together used, various multimeric antigenic determinant sites are feasibly synthesized without template DNA and cloned into vector and moreover its genetic stability can be considerably increased to give recombinant live vaccine with enhanced genetic stability.

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In still further aspect of this invention, there is provided a recombinant single-stranded RNA virus and a recombinant poliovirus vector, characterized in that the recombinant virus is constructed by the method as described above.

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The descriptions for preferred embodiments applied to the present vector correspond to those for the method for enhancing the genetic stability.

For example, the foreign DNA sequence is mutated to provide even distribution of G/C content throughout the overall foreign DNA sequence for improving a genetic stability and the recombinant single-stranded RNA virus

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vector is derived from one selected from the group consisting of a poliovirus recombinant vector, a yellow fever virus vector, a Venezuelan equine encephalitis virus vector, a rubella virus vector and a Cocksackie virus  
5 vector.

In addition, the foreign DNA sequence mutated has the G/C content of more than 40% and is smaller than 450 bp in size.

It is preferred that the recombinant poliovirus vector  
10 is derived from one selected from the group consisting of Sabin type 1, Sabin type 2 and Sabin type 3.

Furthermore, it is preferred that the foreign DNA sequence is inserted into a cloning site containing at least one restriction cleavage site artificially formed at  
15 the 5'-end of the recombinant poliovirus vector. More preferably, the cloning site further comprises at its 3'-direction a protease cleavage site recognized by 3C-protease encoded by a poliovirus genome.

Most preferably, the recombinant Sabin type 1 vector  
20 comprises the foreign DNA sequence mutated according to the above-described method that is inserted into the multiple cloning site depicted in Fig. 1.

As described previously, the common descriptions between the methods and the recombinant virus of this  
25 invention are abbreviated in order to avoid the complexity of this specification leading to undue multiplicity.

The present invention is also drawn to a vaccine

composition comprising chimeric polioviruses isolated from host cells, e.g., HeLa cells transfected with the present poliovirus vector. In preferred embodiment, the chimeric poliovirus comprises a fused antigenic determinant site of  
5 infectious virus. The vaccine composition of this invention comprises a pharmaceutically acceptable carrier or diluent. For example, the chimeric poliovirus prepared according to this invention may be stabilized in solution containing  $MgCl_2$ , sucrose and phosphate. The vaccine  
10 composition of this invention may be administered orally or parenterally. The oral administration is the most preferable mode for the present compositions. The vaccine composition may be administered in the dosage known to one skilled in the art, e.g., less than  $10^6$  TCID<sub>50</sub>.

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The following specific examples are intended to be illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

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**EXAMPLE I: Cloning of Foreign DNA Sequence and Preparation of Recombinant Poliovirus**

**I-1: Preparing Insert DNA**

The DNA fragments coding for the exogeneous antigen  
25 indicated in Table 1 were prepared. The DNA fragments include as monomer, SIV (Simian Immunodeficiency Virus) gag-100 (SEQ ID NO:1), SIV gag-114 (SEQ ID NO:2), SIV p27-



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167 (SEQ ID NO:3), SIV p27-150 (SEQ ID NO:4), SIV env-108  
(SEQ ID NO:5), HIV-1 env-98 (SEQ ID NO:6), HIV-1 env-83  
(SEQ ID NO:7), HIV-1 env-71 (SEQ ID NO:8), HIV-1 env-98/M  
(SEQ ID NO:9), PV(poliovirus) 2-127 (SEQ ID NO:10), PV 2-  
5 118 (SEQ ID NO:11), PV 3-110 (SEQ ID NO:12), HCV core-160  
(SEQ ID NO:13) and HCV core-100 (SEQ ID NO:14); as  
heterodimer, PV 2.3-131 (SEQ ID NO:15), PV 2.3-112 (SEQ ID  
NO:16) and HBV C.S (SEQ ID NO:17); as concatenate multimer,  
HIV-1 mV3 (SEQ ID NO:18) and HIV-1 PND8 (SEQ ID NO:19);  
10 and as designed multimer, OPV (Oral poliovirus)-150 (SEQ  
ID NO:20), OPV-137 (SEQ ID NO:21) and OPV-132 (SEQ ID  
NO:22).

The antigens described above are major antigenic  
determinant sites in the structural proteins of poliovirus  
15 and other pathogenic viruses. In addition, most of  
antigens were designed to have a number of tandemly  
arranged small epitopes, to exert the synergistic immune  
response. However, the present inventors have constrained  
the overall size of the insert to less than 500 bp,  
20 because the upper limit for the stable insert in the wild  
type and other Sabin PV strains poliovirus is around 450-  
600 bp and the genetic stability decreases markedly beyond  
this size. The concatenate multimers were designed to have  
no repeated DNA sequence but the same amino acid sequence  
25 by use of degeneracy of genetic code. The designed  
multimers were synthesized by the ligation-free PCR method  
that has no A/T rich region and repeated DNA sequence, as

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described in Example VI.

I-2: Amplification of Insert DNA

The DNA fragments synthesized above were amplified by the PCR technique. The primers for PCR were designed to have *Sst*II and *Eag*I restriction sites for cloning with RPS-Vax system. The PCR amplification was performed by using the Machine 9700 from Parkin Elmer and PWO DNA polymerase (Cat#: 1644947) from Boehringer Mannheim in the total of 25 cycles: annealing for 30 sec at 47°C, extension for 1 min at 72°C and denaturation for 30 sec at 94°C.

I-3: Preparation of Recombinant Vector and Cloning

The recombinant Sabin 1 cDNA was used in RPS-Vax vector (accession number KCTC 0365BP, denoted as pTZ-PVS-3m) constructed as the disclosure of Jung, H. and Y.-S. Bae *J. Biochem. Mol. Biol.* 31:432-443(1998). The cDNA has a 30-bp polylinker containing an artificial cleavage site for 3C<sup>pro</sup>(AXXQ/G) and a multiple cloning site (*Sst*II, *Hpa*I and *Eag*I restriction sites) (see Fig. 1). Each DNA fragment encoding antigen amplified in Example I-2 was inserted between *Sst*II and *Eag*I restriction sites in the multiple cloning site and amplified in *E. coli* JM109 host cells (STRATAGENE, Cat#:200235).

Among a variety of recombinant Sabin 1 vectors constructed, the vector carrying OPV-150 was denoted as

"RPS/OPV-150", deposited on February 1, 2001 in International Depository Authority, the Korean Collection for Type Cultures and given accession number KCTC 0940BP.

**EXAMPLE II: In vitro Transcription and Transfection**

5 **II-1: In vitro Transcription**

According to Bae et. al method (*Nucleic Acids Res.* 21:2713-2718(1993)), vectors constructed in Example I were linearized with *SalI* and purified by extraction three times with phenol/chloroform, followed by ethanol  
10 precipitation to minimize the contamination of RNase. 0.1  $\mu$ g of linearized plasmid DNA was transcribed in vitro with 5U/ $\mu$ l T7 RNA polymerase in the reaction buffer (40mM Tris-HCl, pH 8.0, 8mM MgCl<sub>2</sub>, 2mM spermidine, 25mM NaCl, 5mM DTT, 1 U/ $\mu$ l RNasin, 2mM dNTP) for 30 min at 37°C. The resultant  
15 was extracted several times with phenol/chloroform and then precipitated with ethanol to give RNA transcript of recombinant virus.

**II-2: Transfection**

Recombinant RNAs were transfected into the cells by a  
20 DEAE-dextran procedure (Van der Welf et al., *PNAS* 83:2330-2334(1986)). 0.2 ml of RNAs (1-2  $\mu$ g) were mixed with the same volume of DEAE-dextran (1 mg/ml in HEPES-buffered saline) and the mixture was coated on monolayers of HeLa cells with 70% confluency, followed by allowing to stand  
25 for 15 min at a room temperature. Then, cells were washed

27

twice with PBS and incubated for 2-3 days in DMEM containing fetal calf serum (FCS). The cytopathic effect was observed according to Jung, H. and Y.-S. Bae *J. Biochem. Mol. Biol.* 31:432-443(1998), demonstrating that all recombinants can produce replication-competent progeny viruses. In addition, one step growth characteristics and kinetics of RNA synthesis of the recombinants were found to be similar to those of the Sabin 1 poliovirus, suggesting that the foreign insert was correctly expressed and its presence did not interfere with the basic viral functions.

Fig. 2 shows the procedures for preparing chimeric poliovirus and recombinant poliovirus vector with fused mV3 or PND regions of HIV.

The foreign antigens expressed in proliferation of chimeric poliovirus were confirmed by Western blotting. In most cases, the Western analyses showed a single prominent band of a correctly processed polypeptide accompanied by minor bands of the higher molecular weight. The minor bands were likely to correspond to partially processed fusion proteins, suggesting that 3C<sup>Pro</sup> protease is not completely efficient in cleavage at this artificial site, probably due to the sub-optimal environment for the enzyme reaction.

### EXAMPLE III: Evaluation of Genetic Stability by RT-PCR

Each chimeric poliovirus generated in Examples described

above was consecutively introduced into the HeLa cells and incubated for 18 hr at 37°C. In each passage, HeLa cell monolayers were infected with the recombinant virus at an MOI of 10, and then the polioviruses were harvested, followed by phenol-chloroform extraction and ethanol precipitation to obtain total RNA. Then, 10 µg of RNA were mixed with 1 µg of primer (GGTAGAACCACCATACGC, 797-814/antisense) and denatured for 10 min at 70°C. Thereafter, the resultant was transferred to ice and the reverse transcriptase reaction solution (50 mM Tris-HCl, pH 8.3, 65 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM NTP mixture, 20 units RNasin) and 200 units of MMLV (moloney Murine Leukemia virus) reverse transcriptase (Promega) were added, followed by incubation for 60 min at 42°C to prepare cDNA. After the completion of reaction, the resultant was incubated for 3 min at 100°C to inactivate enzyme. PCR was then performed using the cDNA prepared as template and Sabin 1 primers (CATTGAGTGTGTTTACTC: 680-697/sense and GGTAGAACCACCATACGC: 797-814/antisense) to amplify the polioviral genome containing inset sequence. PCR was performed with the machine 9700 purchased from Perkin Elmer and Taq polymerase for 25 cycles at 94°C for 30 sec (denaturation), 45°C for 30 sec (annealing), and 72°C for 45 sec (extension). The PCR products amplified were analyzed on agarose gel.

In this Example, the genetic stability at serial passage to twelve was observed. Table 1 represents the

cycle number of passage showing the genetic stability. Figs. 3a (PV 2-118), 3b (PV 2.3-131) and 3c (HIV-1 mV3) show RT-PCR analysis demonstrating a genetic stability of recombinant RPS-Vax. In Fig. 3, lane M represents 100 bp  
5 DNA size marker, lanes S, R and C represent RT-PCR analysis of Sabin type 1, recombinant RPS-Vax and insert-containing recombinant RPS-Vax, respectively.

As shown in Fig. 3a, PV 2-118 fused to RPS-Vax was retained completely until 12<sup>th</sup> cycle of the progressive  
10 passage. As indicated in Table 1, this degree of stability was observed in total 14 out of 22 different recombinants. In addition, as shown in Fig. 3b, RPS-Vax-fused PV 2.3-131, that is a hetero dimer containing a major antigenic  
15 determinant site in VP1 protein of type 2 (Lancing) and type 3 (Leon) among wild types of poliovirus, displayed the intact insert until the 12<sup>th</sup> passage but its genetic stability slowly decreased after the 8<sup>th</sup> passage as evidenced by gradual accumulation of a minor band of the smaller molecular weight. As shown in Fig. 3c, RPS-Vax-  
20 fused HIV-1 mV3, that is a mulimer containing a major antigenic determinant site, V3 region in gp120 of HIV-1, showed lower genetic stability and after 8<sup>th</sup> passage, an intact insert was no longer observed. In addition, HIV-1 PND8, that contains repeated nucleotide sequence as HIV-1  
25 mV3, showed a similar genetic stability to HIV-1 mV3.

As represented in Table 1 and Fig. 3, the insert

sequences exhibiting the highest genetic stability (e.g., PV 2-118) are less than about 450 bp in size and show even G/C distribution with G/C content of more than 40. It is estimated that such sequence would form a compact RNA conformation and readily encapsidated into the rigid viral capsid.

On the other hand, the inserts with the G/C content less than 30% seem to be genetically unstable without regard to the size (e.g., HIV-1 env-98 and HIV env-71). In this situation, even a small RNA insert will be loosely packaged so that it is hardly confined within the limited space inside the capsid. With regard to HCV core-160, while its G/C content is 62.3%, its genetic stability decreased markedly due to its size, 480 bp that is beyond the upper limit of acceptable package size into capsid.

The relationship between G/C content and genetic stability may be applicable when G/C distribution is homologous in the entire region. If the G/C content varies considerably from one region to another, the spatial extent of a whole RNA would be more related to the G/C content of the local region than the overall value. Moreover, the detailed feature within local regions is an important factor because the short-range interaction between neighboring bases is important in formation of secondary structures of RNA, which provide a framework for the tertiary structures. Therefore, the occurrence and

distribution of the specific local sequences should be taken into account, in order to assess the spatial extent of the RNA more precisely.

The results of genetic stability of HIV-1 env-98, HIV-  
5 1 env-83, HIV-1 env-71 and HIV-1 env-98/M, shown in Table 1, Figs. 4a-4c and Fig. 5, confirm the descriptions above. In Fig. 5, lane M represents 100 bp DNA size marker, lanes S, R and C represent RT-PCR analysis of Sabin type 1 and recombinant RPS-Vax and insert-containing recombinant RPS-  
10 Vax, respectively.

As shown in the bottom panel of Fig. 5, RT-PCR analysis showed the truncated fragments of HIV-1 env-98 and HIV-1 env-71, which was subjected to DNA sequencing with dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci.*,  
15 74:5463 (1977)) in order to find out whether this deletion took place in the sequence-specific manner. It was identified that regions of nucleotide 165-261 and 142-264 were deleted from HIV-1 env-98 and HIV-1 env-71, respectively, implying that the region between 165 and 261  
20 is a common deletion site.

The present inventors noticed that the terminal sequence that commonly present in the 3'-end of HIV-1 env-98 and HIV-1 env-71, but not HIV-1 env-83, are extremely A/T rich (see Figs. 4a and 4b). This finding prompted us to  
25 speculate that A/T rich sequence would be potential cause for the marked genetic instability.

The present inventors introduced multiple silent



32

mutations into 13 sites in A/T rich region (nucleotide 250-294) to increase G/C content (see Fig. 4b). The primers (AGTTCAGGAACAAGACCATCGCCCGGCGGTATTA: 1058-1080/sense and TCTCCCTAAGCTTGATCACTATCTGTTGTAAAGTG: 1057-1023/antisense) that have substituted G and C bases for 13 A and T bases were prepared and PCR amplification was performed for HIV-1 env-98/M. The PCR amplification was performed by using the Machine 9700 from Perkin Elmer and PWO DNA polymerase from Boehringer Mannheim in the total of 25 cycles: denaturation for 30 sec at 94°C, annealing for 30 sec at 43°C and extension for 2 min at 72°C. The DNA prepared was introduced into RPS-Vax and transcribed in vitro, followed by transfection to produce replication-competent progeny viruses. Fig. 4b shows that these substitutions increased the G/C content of A/T rich region up to 46.7% from 20% and Fig. 4c represents even distribution of G/C in insert sequence ascribed to these substitutions.

The genetic stability of HIV-1 env-98/M was assessed by RT-PCR as described above and as a result, the complete stability was found throughout the passage (see Fig. 5). This remarkable elevation of the stability indicates clearly that the local A/T rich sequence destabilizes the overall RNA structure and promotes the site-specific deletion of the neighboring region. It also demonstrates that the genetic stability can be manipulated by adjusting the global G/C content and distribution of the insert.

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TABLE 1

Foreign insert DNA	Insert DNA size (bp)	G/C content of insert DNA <sup>a</sup> (%)	$\Delta G$ of insert DNA <sup>b</sup> (kcal/mole)	Stable passage of chimeric poliovirus (number)
SIV gag-100	300	54.3	-160.6	> 12
SIV gag-114	342	44.7	-97.0	> 12
SIV p27-167	501	43.7	-92.6	5
SIV p27-150	450	43.8	-102.3	> 12
SIV env-108	324	35.4	-83.8	6
HIV-1 env-98	294	30.6	-56.2	2
HIV-1 env-83	249	32.5	-48.9	> 12
HIV-1 env-71	213	30.0	-36.5	4
HIV-1 env-98/M	294	34.7	-65.0	> 12
PV 2-127	381	47.5	-113.1	> 12
PV 2-118	354	44.6	-103.9	> 12
PV 2-110	330	50.0	-110.4	> 12
HCV core-160	480	62.3	-196.3	3
HCV core-100	300	60.3	-172.5	> 12
PV 2.3-131	393	48.0	-106.2	7
PV 2.3-112	336	43.8	-110.2	> 12
HEV C.S.	306	46.4	-105.2	> 12
HIV-1 mV3	360	33.9	-58.1	3
HIV-1 RND8	240	43.3	-76.8	9
OPV-150	450	58.4	-138.9	> 12
OPV-137	411	58.4	-132.9	> 12
OPV-132	396	58.8	-147.6	> 12

<sup>a</sup> G/C-contents of inserts counted by DNASIS program (set the window size-9)

<sup>b</sup>  $\Delta G$  indicates the free energy of the inserts RNA in secondary structure predicted by DNASIS program (set the maximum bulge and interior loop size-30)

#### EXAMPLE IV: Evaluation of Genetic Stability by Western Blotting

5 HeLa cells were transfected with Sabin type 1 and

34

recombinant viruses (MOI = 10) and incubated for 24 hr at 37°C, followed by harvesting cells. The cells were resuspended with PBS and mixed with the same volume of 2x sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 1%  $\beta$ -mercaptoethanol, bromophenol blue, and 0.01 mg/ml xylene cyanol), after which the resuspension was boiled for 10 min and centrifuged to discard nucleus. The total lysates were applied to a SDS-12% PAGE. Then the resultant was transblotted to a nitrocellulose membrane using a semi-dry gel transfer system (Bio-Rad). Blotted membranes were firstly incubated with monkey anti-SIV<sub>mac239</sub> sera kindly provided from German Primate Center and then secondly incubated with alkaline phosphatase (AP)-conjugated anti-IgG-AP (Sigma). The membranes were transferred to a reaction solution containing NBT/BCIP (Sigma) for alkaline phosphatase and the bands with developed color were observed. Figs. 6a and 6b are photograph showing RT-PCR analysis demonstrating a genetic stability and Western blot analysis demonstrating a protein stability of SIV p27-150 and SIV p27-167, respectively. As shown in Fig. 6a, SIV p27-150 showed a complete genetic stability, whereas SIV p27-167 showed considerable-decreased genetic stability after 6<sup>th</sup> passage. In addition, SIV p27-167 showed considerable-decreased protein expression along with decreased genetic stability.

**EXAMPLE V: Recovery of Viruses from the inoculated Tg-PVR**

### Mice

The optimal inoculation conditions of Sabin 1 type poliovirus of this invention to Tg-PVR mice were established. With respect to the inoculation route, intracerebral, intramuscular and intravenous routes were revealed to be effective. The optimal age of mice (4 weeks), the inoculation dose ( $1 \times 10^7$  PFU), the minimal time for eliciting immune response (more than 4 weeks) and the time when viruses proliferated can be isolated (within 5 days) were revealed. The experiment was standardized on the basis of the optimal conditions elucidated. The mice were inoculated intracerebrally according to the standardized conditions with the suspension of chimeric poliovirus produced using SIV p27-150 or SIV p27-167-fused RPS-Vax. A microsyringe and specially designed 26/30 gauge needles were used for minimizing the damage due to inoculation. Mice were sacrificed daily after inoculation. The spleen was separated from each mouse, and homogenized. The supernatants from homogenates were transferred into HeLa cell monolayers to recover the virus passed in vivo. They were tested for genomic integrity by RT-PCR as described in Example III. As represented in Fig. 7, SIV p27-150 showed a genetic stability during 4<sup>th</sup> passage whereas SIV p27-167 exhibited considerable-reduced genetic stability from 2<sup>nd</sup> passage. These results correspond to those of Fig. 6 using HeLa cells.

The results imply that the genetic stability of

recombinant poliovirus according to this invention can be maintained *in vivo* as well as *ex vivo*.

**EXAMPLE VI: Evaluation of Genetic Stability of Artificial  
5 Epitope Multimer**

It is general to construct a multimeric epitope for improving antigenicity of vaccine. However, the insert containing repeated sequence is very likely to be deleted (e.g., HIV-1 mV3; Fig. 3c).

10 The genetic stability of any given insert less than 450 bp, as revealed in Examples above, can be promoted by adjusting G/C content to above 40% along with even distribution of G/C. Therefore, the insert sequences with repetitive sequence are required for its codon to be adjusted  
15 according to the rule of this invention: first, avoid the less used codon in human as a host for poliovirus, second, use the high G/C content among the highly preferred ones, and third, minimize the local repeat such as A/T rich sequence for the whole insert.

20

**VI-1: Rapid Synthesis of DNA with Ligation-free PCR**

OPV-150, OPV-137 and OPV-132 were synthesized by ligation-free PCR according the rule described previously. In OPV-150, the DNA sequence encoding antigenic  
25 determinant sites with repeated neutralizing epitopes of Lansing poliovirus and Leon poliovirus was designed to avoid repeated sequences on the basis of our G/C rules.

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Thereafter, for synthesizing the DNA sequence designed by ligation-free PCR, 8 DNA fragments (1,3,5,7/sense and 2,4,6,8/antisense) containing cloning sites (*Sst*II/1 and *Eag*I/2 cleavage sites) and serving as both primer and  
5 template were synthesized (Bionix Inc., Korea) and purified in PAGE (see Fig. 9). Each primer has 15-mer at its 5'- or 3'-end capable of annealing to its complementary primer. Among 8 primers, the primers corresponding to both ends which are used in amplification  
10 of concatemeric multimer with desired size and containing restriction sites for cloning were added in amount of 200 ng (where the volume of reactant was 50  $\mu$ l) and other 10 primers only for maturation were added in amount of 50 ng and mixed. In other words, the mixing ratio was adjusted  
15 to 1:4. The resultant was thermally cycled for 5-10 cycles: denaturation for 30 sec at 94°C, annealing for 30 sec at 35-40°C and PCR extension for 1 min at 72. Thereafter, the resultant was thermally cycled for 20-25  
20 cycles: denaturation for 30 sec at 94°C, annealing and extension for 1 min at 72 to amplify concatenate-multimers with desired size. The PCR amplification was performed by using the Machine 9700 from Perkin Elmer and PWO DNA polymerase (Cat#: 1644947) from Boehringer Mannheim. Fig.  
10 illustrates the products of ligation-free PCR. In Fig.  
25 10, lane M represents 100 bp size marker, lane 1 show the product using primers 1, 2, 3 and 4, lane 2 the product using primers 1, 2, 3, 4, 5 and 6, and lane 3 the product

using primers 1, 2, 3, 4, 5, 6, 7 and 8. As shown in Fig. 10, the DNA sequence encoding OPV-150 was synthesized using ligation-free PCR of this invention.

5 VI-2: Evaluation of Genetic Stability

The genetic stability of OPV-150, OPV-137 and OPV-132 was evaluated and revealed to be maintained up to 12<sup>th</sup> passage, as indicated in Table 1. Fig. 11a represents a photograph showing RT-PCR analysis demonstrating a genetic  
10 stability of OPV-150; and Fig. 11b is a photograph showing Western blot analysis demonstrating a protein stability. The primary antibody used was BSA-conjugated anti-PV2 and antibody obtained by immunization of BALB/C mice with PV3 epitope peptide, that was prepared by the inventors. As  
15 represented in Figs. 11a and 11b, its was demonstrated that OPV-150 showed the stability in both genomic level and protein level.

[EFFECTS OF THIS INVENTION]

20 The present invention provides a method for improving a genetic stability of a foreign insert DNA sequence in a recombinant single-stranded RNA virus vector. Furthermore, this invention provides a method for improving a genetic stability of a foreign insert DNA sequence in a  
25 recombinant poliovirus vector. In addition to this, this invention provides a method for preparing a DNA molecule using ligation-free PCR method. The present method

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provides a vector comprising more various antigenic  
determinant sites compared to the conventional poliovirus  
vector technology and a method for improving significantly  
a genetic stability of a foreign sequence, thereby  
5 permitting to enforce the applicability of poliovirus  
vector as recombinant live vaccine.



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What is claimed is:

1. A method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant single-stranded RNA virus vector, which comprises  
5 performing a mutagenesis of the foreign insert nucleotide sequence to provide even distribution of G/C content throughout the overall foreign DNA sequence.
2. The method according to claim 1, wherein the  
10 recombinant single-stranded RNA virus vector is derived from one selected from the group consisting of a poliovirus recombinant vector, a yellow fever virus vector, a Venezuelan equine encephalitis virus vector, a rubella virus vector and a Coxsackie virus vector.
- 15 3. A method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant poliovirus vector, which comprises performing a mutagenesis of the foreign insert nucleotide sequence to  
20 provide even distribution of G/C content throughout the overall foreign DNA sequence.
4. The method according to claim 1 or 3, wherein the mutagenesis to provide even distribution of G/C content  
25 throughout the overall foreign DNA sequence is performed by increasing G/C content of the foreign DNA sequence.

41

5. The method according to claim 4, wherein the mutagenesis renders the foreign DNA sequence to have the G/C content of more than 40%.

5 6. The method according to claim 1 or 3, wherein the mutagenesis of the foreign DNA sequence to provide even distribution of G/C content is performed by increasing G/C content of local A/T-rich region in the foreign DNA sequence.

10

7. The method according to claim 1 or 3, wherein the mutagenesis is performed by silent mutations.

8. The method according to claim 1 or 3, wherein the  
15 foreign DNA sequence is smaller than 450 bp in size.

9. The method according to claim 2 or 3, wherein the recombinant poliovirus vector is one selected from the group consisting of poliovirus type 1, poliovirus type 2  
20 and poliovirus type 3.

10. The method according to claim 2 or 3, wherein the poliovirus is one selected from the group consisting of Sabin type 1, Sabin type 2 and Sabin type 3.

25

11. The method according to claim 1 or 3, wherein the foreign DNA sequence encodes an antigenic determinant site

of an infectious virus selected from HIV, HCV, HBV, HPV, HSV, rotavirus, influenza virus and epidemic hemorrhagic fever virus.

5 12. The method according to claim 3, wherein the foreign DNA sequence is inserted into the 5'-end of the recombinant poliovirus vector.

10 13. The method according to claim 12, wherein the foreign DNA sequence is inserted into a cloning site containing at least one restriction cleavage site artificially formed at the 5'-end of the recombinant poliovirus vector.

15 14. The method according to claim 13, wherein the cloning site further comprises at its 3'-direction a protease cleavage site recognized by 3C-protease encoded by a poliovirus genome.

20 15. A method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant Sabin type 1 poliovirus vector comprising (a) a genomic nucleotide sequence of poliovirus; (b) a cloning site containing restriction cleavage sites artificially formed at the 5'-end of the genomic nucleotide sequence; and (c)  
25 a 3C-protease cleavage site formed between the genomic nucleotide sequence and the cloning site, which comprises performing a mutagenesis of the foreign DNA sequence

inserted into the cloning site in order to provide even distribution of G/C content throughout the overall foreign DNA sequence and to have the G/C content of more than 40%.

5 16. A method for improving a genetic stability of a foreign insert nucleotide sequence in a recombinant Sabin type 1 poliovirus vector comprising (a) a genomic nucleotide sequence of poliovirus; (b) a multiple cloning site containing restriction cleavage sites artificially  
10 formed at the 5'-end of the genomic nucleotide sequence; and (c) a 3C-protease cleavage site formed between the genomic nucleotide sequence and the cloning site, which comprises performing a mutagenesis of the foreign DNA sequence which is inserted into the multiple cloning site  
15 and encodes a multimeric antigen determinant site containing repeated amino acid sequences in order to provide even distribution of G/C content throughout the overall foreign DNA sequence and to avoid repeated nucleotide sequences by use of degeneracy of genetic code.

20

17. The method according to claim 16, wherein the mutated foreign DNA sequence encoding multimeric antigen determinant site is prepared by a ligation-free PCR method which comprises the steps of:

25 (i) preparing a plurality of DNA fragment serving as both template and primer and having the following characteristics: (a) the plural primers are designed

44

by dividing the entire DNA sequence encoding the multimeric antigen determinant site into several fragments with suitable size; (b) the 5'- and/or 3'-ends of the plural primers comprise a complementary sequence to the terminal sequence of other primer; (c) the complementary sequence is 8-20 mer in size and has G/C content of more than 35%; (d) the primers corresponding to both ends in amplification step have a cloning site containing restriction cleavage site;

5 (ii) mixing the primers such that the concentration ratio of the primers corresponding to both ends in amplification step to the other primers is 1:3-1:8;

10 (iii) preparing a full length of the foreign DNA sequence with desired size encoding multimeric antigen determinant site by PCR for 20-40 sec at 92-96°C (denaturation), for 20-40 sec at 25-40°C (annealing) and for 30-55 sec at 68-75°C (extension); and

15 (iv) amplifying the prepared full length of the foreign DNA sequence by PCR for 20-40 sec at 92-96°C (denaturation) and 40 sec - 1 min 10 sec at 68-75°C (annealing and extension).

20

18. A method for amplifying a DNA sequence using a ligation-free PCR method, which comprises the steps of:

25 (i) preparing a plurality of DNA fragment serving as both template and primer and having the following characteristics: (a) the plural primers are designed

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by dividing the entire DNA sequence of interest into several fragments into several fragments with suitable size; (b) the 5'- and/or 3'-ends of the plural primers comprise a complementary sequence to the terminal sequence of other primer;

(ii) mixing the primers such that the primers corresponding to both ends which is used in amplification step has a higher concentration than the other primers;

(iii) preparing a full length of the foreign DNA sequence with desired size encoding multimeric antigen determinant site by PCR for 20-40 sec at 92-96°C (denaturation), for 20-40 sec at 25-40°C (annealing) and for 30-55 sec at 68-75°C (extension); and

(iv) amplifying the prepared full length of the foreign DNA sequence by PCR for 20-40 sec at 92-96°C (denaturation) and 40 sec - 1 min 10 sec at 68-75°C (annealing and extension).

19. The method according to claim 18, wherein the concentration ratio of the primers corresponding to both ends in amplification step to the other primers is 1:3-1:8.

20. The method according to claim 18, wherein the complementary sequence at the the 5'- and/or 3'-ends of the primers is 8-20 mer in size and has G/C content of more than 35%.

21. The method according to claim 18, wherein the primers corresponding to both ends in amplification step have a cloning site containing restriction cleavage site.

5 22. A recombinant single-stranded RNA virus vector comprising a foreign DNA sequence, characterized in that the foreign DNA sequence is mutated to provide even distribution of G/C content throughout the overall foreign DNA sequence for improving a genetic stability and the  
10 recombinant single-stranded RNA virus vector is derived from one selected from the group consisting of a poliovirus recombinant vector, a yellow fever virus vector, a Venezuelan equine encephalitis virus vector, a rubella virus vector and a Coxsackie virus vector.

15

23. The recombinant single-stranded RNA virus vector according to claim 22, wherein the foreign DNA sequence mutated has the G/C content of more than 40% and is smaller than 450 bp in size.

20

24. A recombinant poliovirus vector comprising a foreign DNA sequence, characterized in that the foreign DNA sequence is mutated to provide even distribution of G/C content throughout the overall foreign DNA sequence for  
25 improving a genetic stability and the recombinant poliovirus vector is derived from one selected from the group consisting of poliovirus type 1, poliovirus type 2

47

and poliovirus type 3.

25. The recombinant poliovirus vector according to claim 24, wherein the foreign DNA sequence mutated has the G/C  
5 content of more than 40%.

26. The recombinant poliovirus vector according to claim 24 or 25, wherein the foreign DNA sequence is smaller than 450 bp in size

10

27. The recombinant poliovirus vector according to claim 24, wherein the recombinant poliovirus vector is derived from one selected from the group consisting of Sabin type 1, Sabin type 2 and Sabin type 3.

15

28. The recombinant poliovirus vector according to claim 24, wherein the foreign DNA sequence is inserted into a cloning site containing at least one restriction cleavage site artificially formed at the 5'-end of the recombinant  
20 poliovirus vector.

29. The recombinant poliovirus vector according to claim 28, wherein the cloning site further comprises at its 3'-direction a protease cleavage site recognized by 3C-  
25 protease encoded by a poliovirus genome.



Fig. 1

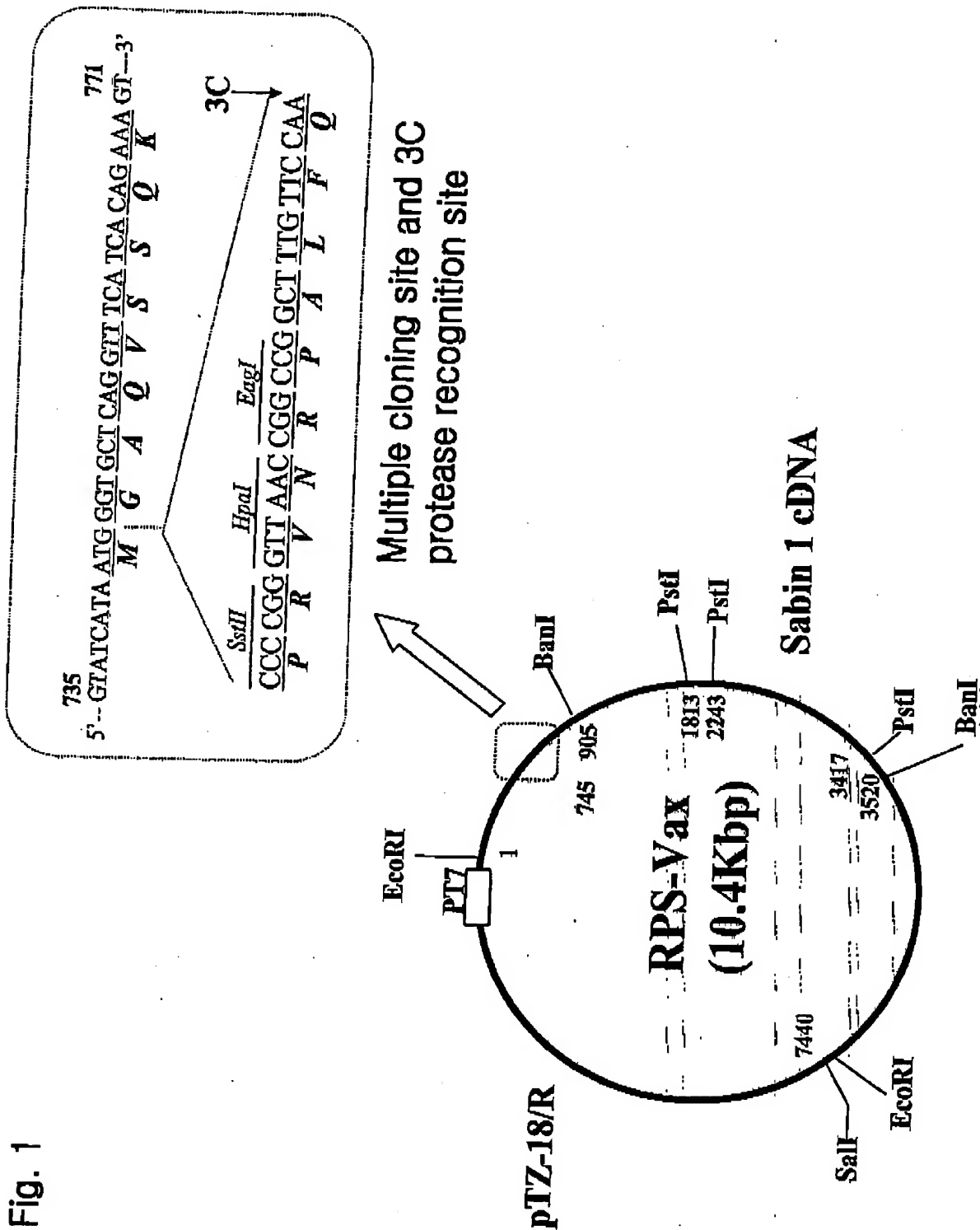


Fig. 2

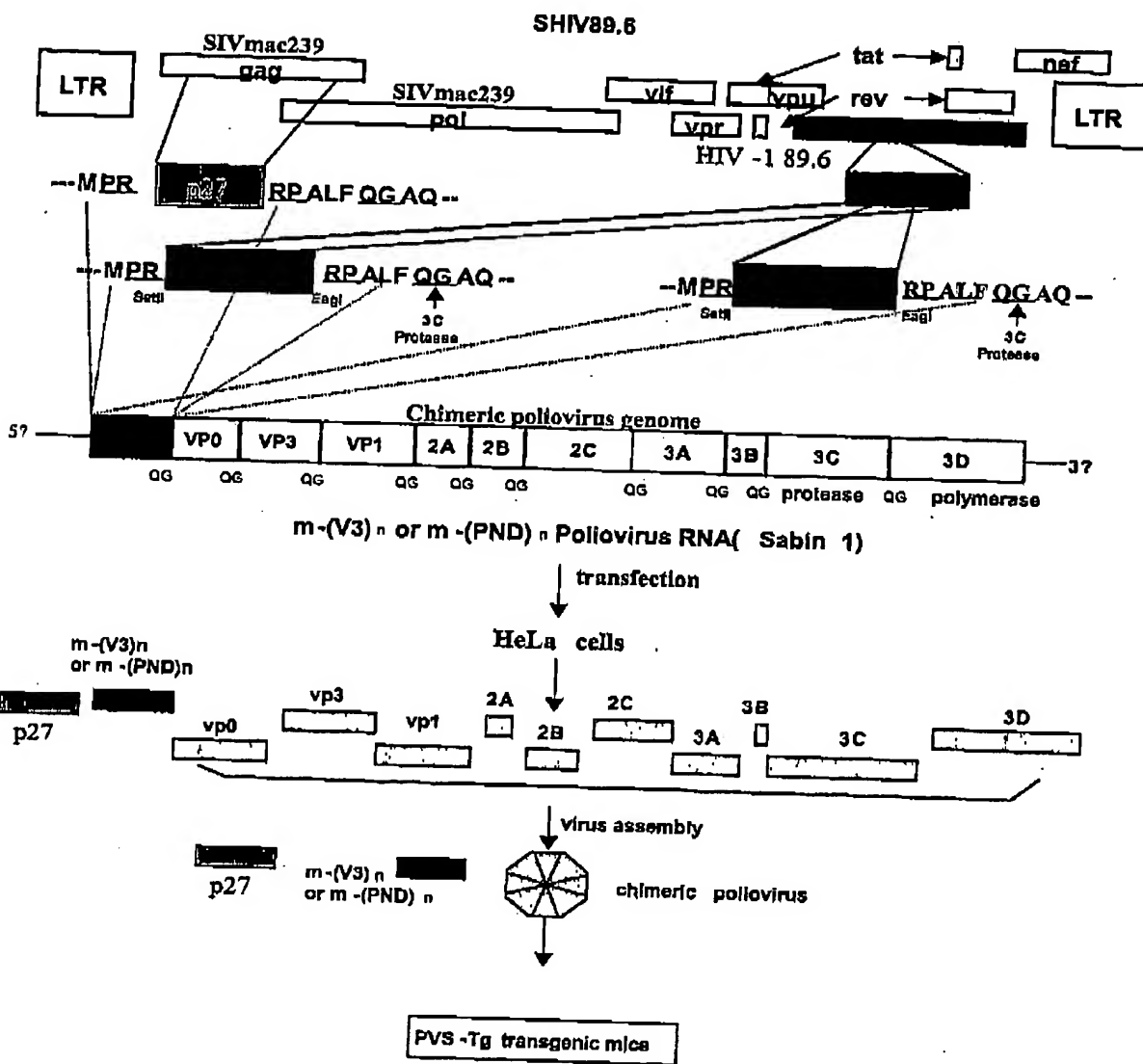


Fig. 3a

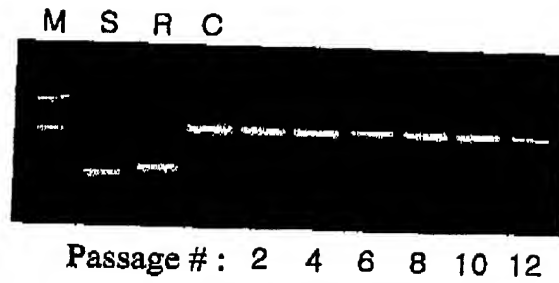


Fig. 3b

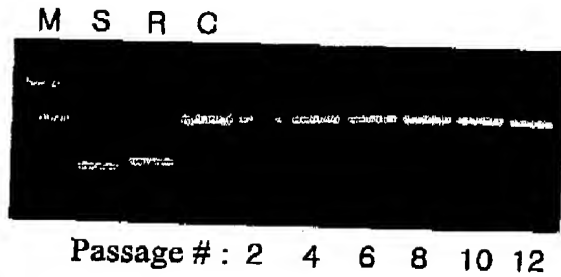
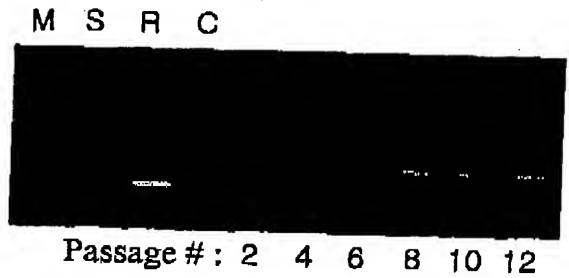


Fig. 3c



	Major deletion site			Base	G/C(%)	$\Delta G$
env-98	1	165	261	294	30.6	-56.2
env-83	1		249	249	32.5	-48.9
env-71	52	142	264	294	30.0	-36.5
env-98/M	1		250	294	34.7	-65.0

Mutagenesis site of A/T rich region

- I V I K L R E K F R N K T I A		<u>G/C(%)</u>	<u>ΔG</u>
ATA GTT ATA AAA TTA AGA GAA AAA TTT AGG AAT AAA ACA ATA GCC		20.0	+0.3
ATA GTG ATCAAG CTT AGG GAGAAG TTC AGG AAC AAGACC ATC GCC			↓
250		46.7	-10.4

Fig. 5

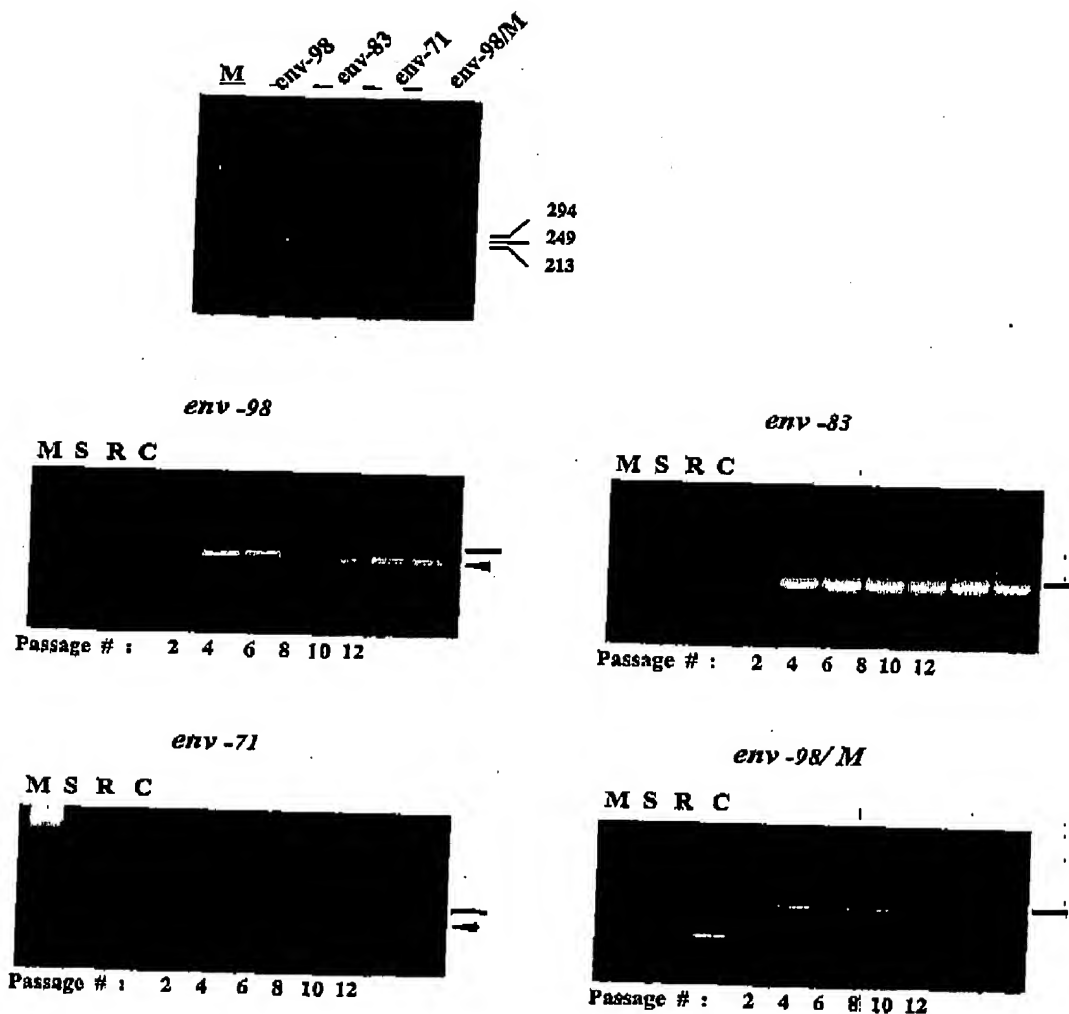


Fig. 6a

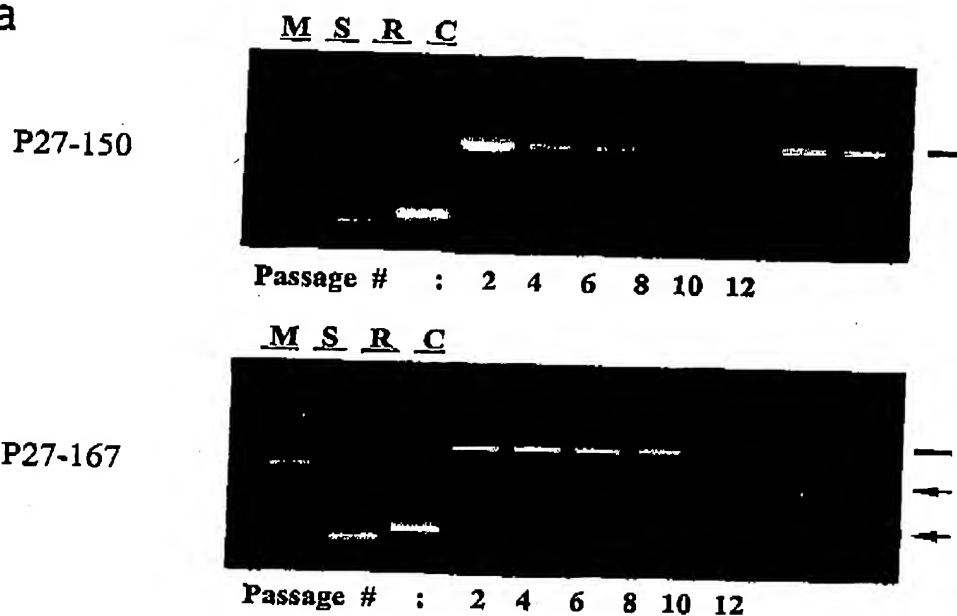


Fig. 6b

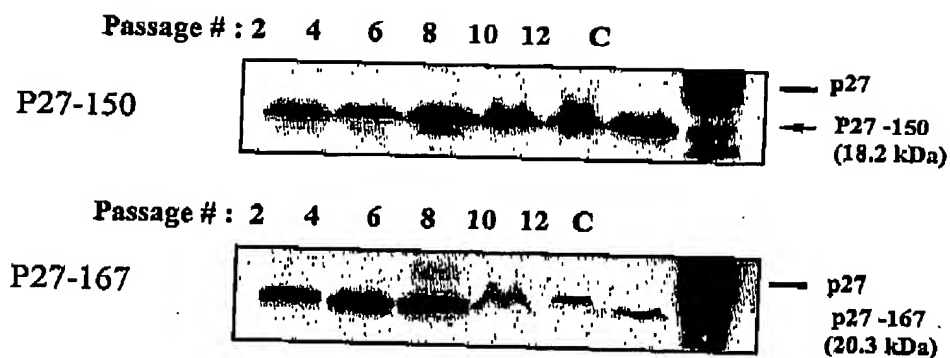


Fig. 7

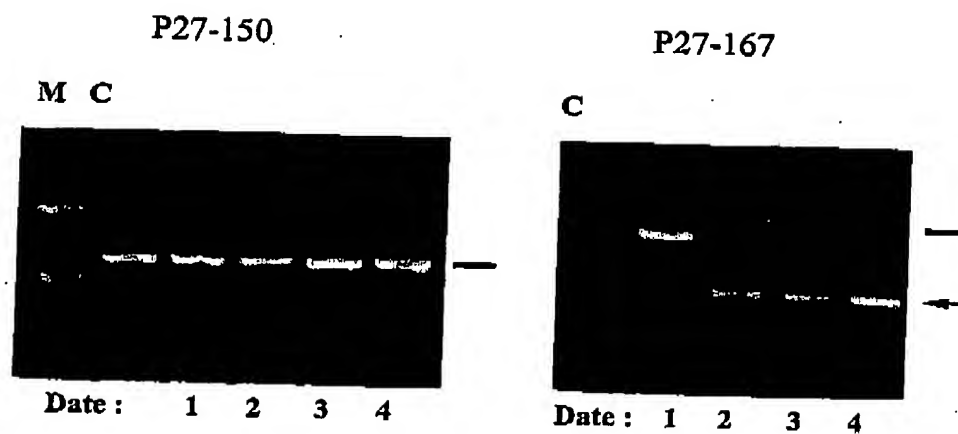


Fig. 8

1	A K A V A A W T L K A A A G Q	15
1	GCT AAG GCC GTT GCA GCC TGG ACC CTG AAA GCC GCT GCA GGC CAA	45
16	A S T E G D C G C P A I I E V	30
46	GCC TCC ACC GAA GGC GAC TGC GGT TGC CCA <u>GCC ATC ATC GAG GTC</u>	90
	GCC ATT ATT GAA GTG	
31	<u>D N D A P T K R A S K L</u> F S E	45
91	GAT AAG GAT GCC CCT ACC AAG CGA GGC AGC AAG CTC TTC AGC GAA	135
	GAT AAT GAT GCT CCA ACA AAG CGT GCC AGT AAA TTA	
46	F E V D <u>N E Q P T T R A Q K</u> L	60
136	TTC GAG GTC GAT <u>AAT GAG CAG CCC ACT ACC CGA GCC CAG AAG</u> CTC	180
	AAT GAA CAA CCA ACC ACC CGG GCA CAG AAA	
61	F A M W R I T Y K <u>D N D A P T</u>	75
181	TTC GGC ATG TGG CGT ATC ACT TAC AAG <u>GAC AAT GAT GCG CCA ACT</u>	225
	GAT AAT GAT GCT CCA ACA	
76	<u>K R A S K L</u> C V R I Y M K P K	90
226	<u>AAG CGC GCA TCT AAA CTG</u> TGC GTC CGA ATC TAC ATG AAG CCC AAG	270
	AAG CGT GCC AGT AAA TTA	
91	H V R C S G C P A I I E V <u>D N</u>	105
271	CAC GTT CGA TGC TCC GGC TGT CCC <u>GCT ATT ATC GAA GTG GAT AAC</u>	315
	GCC ATT ATT GAA GTG GAT AAT	
106	<u>D A P T K R A S K L</u> D N Y Q S	120
316	<u>GAC GCA CCA ACC AAA CGG GCA TCA AAG CTG</u> GAC AAC TAC CAG TCC	360
	GAT GCT CCA ACA AAG CGT GCC AGT AAA TTA	
121	P C A I <u>N E Q P T T R A Q K</u> S	135
361	CCA TGC GCG ATC <u>AAC GAG CAA CCT ACC ACC CGT GCG CAA AAG</u> TCC	405
	AAT GAA CAA CCA ACC ACC CGG GCA CAG AAA	
136	A G C F Y Q T R V V V P S G C	150
406	GCT GGG TGC TTC TAT CAG ACC CGC GTC GTG GTT CCC TCA GGT TGT	450

- : Lansing (wt 2 poliovirus) VP1 neutralizing epitopes.  
 ori - GCC ATT ATT GAA GTG GAT AAT GAT GCT CCA ACA AAG CGT GCC AGT AAA TTA  
   : Leon (wt 3 poliovirus) VP1 neutralizing epitopes  
 ori - AAT GAA CAA CCA ACC ACC CGG GCA CAG AAA CTA  
 — : Coding sequence diversity mediated by application of various codon usage.



Fig. 9

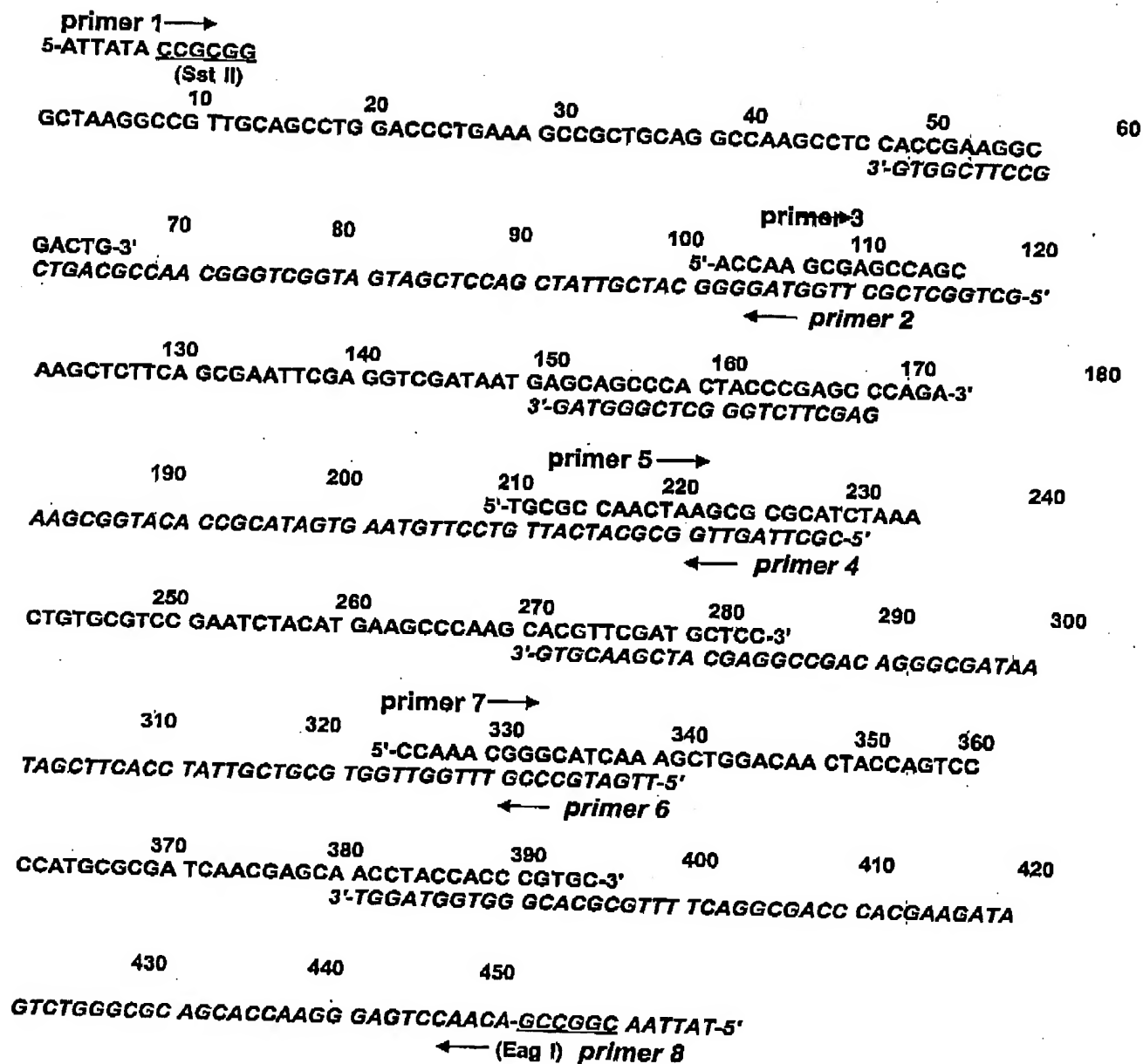


Fig. 10

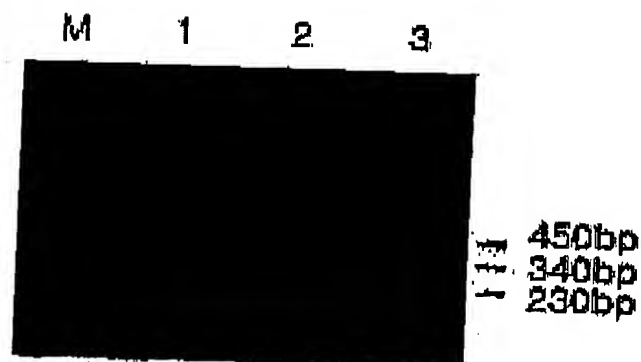


Fig. 11a

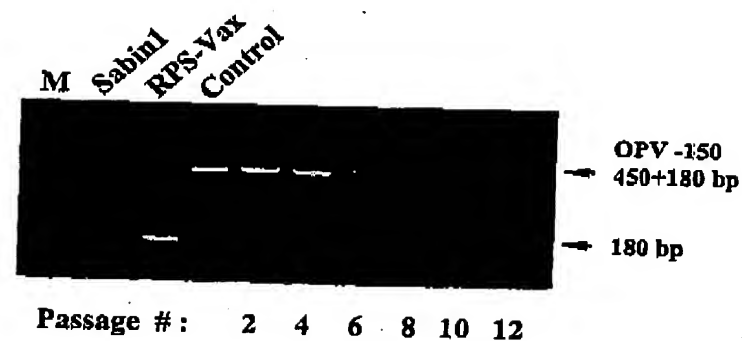
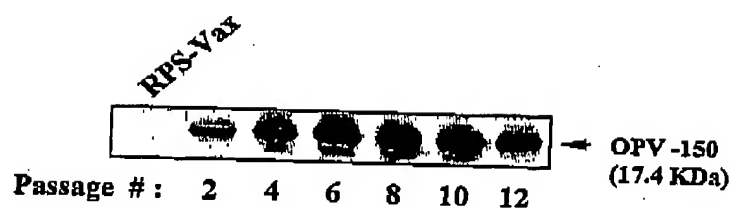


Fig. 11b



1

## 【Sequence Listing】

&lt;110&gt; CreaGene Inc.

<120> Method for Improving a Genetic Stability for the Insert in  
Single-Stranded RNA Virus Recombinant Vectors

&lt;130&gt; CreaGene-1

&lt;160&gt; 22

&lt;170&gt; KopatentIn 1.71

&lt;210&gt; 1

&lt;211&gt; 300

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; SIV gag-100

&lt;400&gt; 1

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agcccgagaa cattaaatgc ctgggtaaaa ttgatagagg aaaagaaatt tggagcagaa      60
gtagtgccag gatttcaggc actgtcagaa ggttgcaccc cctatgacat taatcagatg      120
ttaaattgtg tgggagacca tcaagcggct atgcagatta tcagagatat tataaacgag      180
gaggctgcag attgggactt gcagcaccca caaccagctc cacaacaagg acaacttagg      240
gagccgtcag gatcagatat tgcaggaaca actagttcag tagatgaaca aatccagtgg      300

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&lt;210&gt; 2

&lt;211&gt; 342

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; SIV gag-114

&lt;400&gt; 2

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ccagtacaac aaatagggtg taactatgtc caccigccat taagcccgag aacattaaat      60
gcctgggtaa aattgataga ggaaaagaaa ttggagcag aagtagtgcc aggatitcag      120
gcactgtcag aaggttgcac cccctatgac attaatcaga tgttaaattg tgtgggagac      180
catcaagcgg ctatgcagat tatcagagat attataaacg aggaggctgc agattgggac      240

```

2

ttgcagcacc cacaaccagc tccacaacaa ggacaactta gggagccgtc aggatcagat	300
attgcaggaa caactagttc agtagatgaa caaatccagt gg	342

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 <211> 501  
 <212> DNA  
 <213> Artificial Sequence

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 <223> SIV p27-167

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gcactgtcag aaggttgcac cccctatgac attaatcaga tgttaaattg tgtgggagac	180
catcaagcgg ctatgcagat tatcagagat attataaacg aggaggctgc agattgggac	240
ttgcagcacc cacaaccagc tccacaacaa ggacaactta gggagccgtc aggatcagat	300
attgcaggaa caactagttc agtagatgaa caaatccagt ggatgtacag acaacagaaac	360
cccataccag taggcaacat ttacaggaga tggatccaac tggggttgca aaaatgtgtc	420
agaatgtata acccaacaaa cattctagat gtaaaacaag ggccaaaaga gccatttcag	480
agctatgtag acaggttcta c	501

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 <211> 450  
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 <213> Artificial Sequence

<220>  
 <223> SIV p27-150

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gcactgtcag aaggttgcac cccctatgac attaatcaga tgttaaattg tgtgggagac	180
catcaagcgg ctatgcagat tatcagagat attataaacg aggaggctgc agattgggac	240
ttgcagcacc cacaaccagc tccacaacaa ggacaactta gggagccgtc aggatcagat	300

3

attgcaggaa caactagttc agtagatgaa caaatccagt ggatgtacag acaacagaa 360  
 ccataccag taggcaacat ttacaggaga tggatccaac tggggttgca aaaatgtgtc 420  
 agaattgtata acccaacaaa cattctagat 450

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<220>  
 <223> SIV env-108

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 tgtagaagac caggaaataa gacagtttta ccagtcacca ttaigtctgg attggttttc 180  
 cactcacaac caatcaatga taggccaaag caggcatggt gttggtttgg aggaaaatgg 240  
 aaggatgcaa taaaagaggc gaagcagacc attgtcaaac atcccaggta tactggaact 300  
 aacaatactg ataaaatcaa ttg 324

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 <211> 294  
 <212> DNA  
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 <223> HIV-1 env-98

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 gctaaaacca taatagtaca gctaaatgaa tctgtagtaa ttaattgtac aagaccaaac 120  
 aacaatacaa gaagaagggt atctatagga ccaggagag cattttatgc aagaagaaac 180  
 ataataggag atataagaca agcacattgt aacattagta gagcaaaatg gaataacact 240  
 ttacaacaga tagttataaa attaagagaa aaatttagga ataaaacaat agcc 294

<210> 7  
 <211> 249  
 <212> DNA

4

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; HIV-1 env-83

&lt;400&gt; 7

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 gclaaaacca taatagtaca gctaaatgaa tctgtagtaa ttaattgtac aagacccaac 120  
 aacaatacaa gaagaagggtt atctatagga ccaggagag cattttatgc aagaagaac 180  
 ataataggag atataagaca agcacattgt aacattagta gagcaaaatg gaataacact 240  
 ttacaacag 249

&lt;210&gt; 8

&lt;211&gt; 213

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; HIV-1 env-71

&lt;400&gt; 8

ctaaatgaat ctgtagtaat taattgtaca agacccaaca acaatacaag aagaaggtaa 60  
 tctataggac caggagagc attttatgca agaagnaaca taataggaga tataagacaa 120  
 gcacattgta acattagtag agcaaaatgg aataacactt tacaacagat agttataaaa 180  
 ttaagagaaa aatttaggaa taaaacaata gcc 213

&lt;210&gt; 9

&lt;211&gt; 294

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; HIV-1 env-98/M

&lt;400&gt; 9

ttaaatggca gtctagcaga agaagacata gtaattagat ctgaaaattt cacagacaat 60  
 gctaaaacca taatagtaca gctaaatgaa tctgtagtaa ttaattgtac aagacccaac 120  
 aacaatacaa gaagaagggtt atctatagga ccaggagag cattttatgc aagaagaac 180  
 ataataggag atataagaca agcacattgt aacattagta gagcaaaatg gaataacact 240

5

ttacaacaga tcgtgatcaa gcttcgggag aagttccgga acaagacgat cgcc

294

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 agaggagctt gtgtggccat tattgaagtg gataatgatg ctccaacaag gcgtgccagt 180  
 aaattatttt cagtcggaa gataacttac aaggacacgg ttcagttaag acgtaagttg 240  
 gagttcttta catattcaag gtttgacatg gagttcacct ttgtggttac atccaattat 300  
 accgatgcaa acaatgggca cgcactgaat caagtttacc agataatgta cataccacct 360  
 ggggcaccga tccttgcaa g 381

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 <223> PV 2-118

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 ttacatatt caaggttga catggagttc acctttgtgg ttacatccaa ttataccgat 180  
 gcaaaootg ggcacgcact gaatcaagtt taccagataa tgtacatacc acctggggca 240  
 ccgatccctg gcaagcggaa tgattacaca tggcaaacgt catctaacc atcagtgttt 300  
 tacacttacg gggcacctcc agctagaata tcagtgcctt acgtgggcat tgcc 354

<210> 12  
 <211> 330

6

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<220>  
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gcgtgcgtcg ctattattga ggtggacaat gaacaaccaa ccacccgggc acagaaacta 120  
tttgccatgt ggcgcatcac atacaaagat acagtgcagt tgcgccgtaa gttggagttt 180  
ttcacatact ctcgttttga catggaattc accttcgtgg taaccgcaa cttcaccaac 240  
gctaataatg ggcatgcact caaccaggtg taccagataa tgtacatccc cccaggggca 300  
cccacaccaa agtcatggga cgactacact 330

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<212> DNA  
<213> Artificial Sequence

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<223> HCV core-160

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ggccccagggt tgggtgtgcg cgcgactagg aagacttccg agcggtcgca acctcgtgga 180  
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tacctttggc cctctatgg caatgagggt ctgggatggg caggatggct cctgtcacc 300  
cggggtcttc ggcctagtgt gggccccaca gacccccggc gtaggtcgcg taatttgggt 360  
aaggatcatg atactctcac atgcggcttc gccgacctca tgggtacat tccgtcgtc 420  
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<210> 14  
<211> 300  
<212> DNA  
<213> Artificial Sequence



7

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&lt;223&gt; HCV core-100

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ggccccaggt tgggtgtcgc cgcgactagg aagacticcg agcggtcgca acctcgtgga 180  
aggcgacagc ctatcccaa ggctcgcaa cccgaggga ggacctgggc tcagcccggg 240  
taaccttggc cctctatgg caatgagggt ctgggatggg caggatggct cctgtcacc 300  
300

&lt;210&gt; 15

&lt;211&gt; 399

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PV 2.3-131

&lt;400&gt; 15

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agaggagcct gtgtggccat tattgaagtg gataatgatg ctccaacaag gcgtgccagt 180  
aaattatitt cagtctggaa gataactgaa ttcgagtcga caatagaatc attcttcgca 240  
cgccggcggt gcgtcgctat tattgaggtg gacaatgaac aaccaaccac ccgggcacag 300  
aaactatttg ccatgtggcg cattacatac aaagatacag tgcagttgcg ccgtaagt 360  
gagtttttca catactctcg ttttgacatg gaattcacc 399

&lt;210&gt; 16

&lt;211&gt; 336

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PV 2.3-112

&lt;400&gt; 16

8

gcttgtgtgg ccattattga agtggataat gatgctccaa caaggcgtgc cagtaaatta 60  
 ttttcagtct ggaagataac ttacaaggac accgttcagt taagacgtaa gtggagttc 120  
 ttacatatt caaggtttga catggagttc acctttgttg ttacaggatc cgcgtgcgtc 180  
 getattattg aggtggacaa tgaacaacca accacccggg cacagaaact atttgccatg 240  
 tggcgatta catacaaaga tacagtgcag ttgcgccgta agttggagtt tttcacatac 300  
 tctcgttttg acatggaatt caccttcgtg gtaacc 336

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 <211> 306  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> HBV C.S

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 tcaacacttc cggaaactac tgttgttga gaattcccag gatcatcaac caccagcacg 180  
 ggaccatgca agacttgca agctcctgct caaggaaact ctatgtttcc ctcatgttgc 240  
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 gcaaaa 306

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 <211> 360  
 <212> DNA  
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<220>  
 <223> HIV-1 mV3

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 ttcatataat gtacaagacc caacaacaat acaagaagaa ggttatctat aggaccaggg 180  
 agagcatttt atgcaagaag aaacataata ggagatataa gacaagcaca ttgtaacatt 240

9

ctgcagatta attgtacaag acccaacaac aatacaagaa gaaggttatc tataggacca 300

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360

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<211> 240  
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<220>  
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<212> DNA  
<213> Artificial Sequence

<220>  
<223> OPV-150

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aagctcttca gcgaattcga ggtcgataat gagcagccca ctacccgagc ccagaagctc 180  
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cagacccgcg tcgtggttcc ctgaggttgt 450

10

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<212> DNA  
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<223> OPV-137

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aagctggaca actaccagtc cccatgcgcg atcaacgagc aacctaccac cctgctcgaa 360  
aagtcgcgtg ggtgcttcta tcagaccgcg gtcgtggttc cctcaggttg t 411

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<212> DNA  
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<220>  
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aagctcttcg ccatgtggcg tatcacttac aaggacaatg atgcgccaac taagcgcgca 180  
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